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14. ABSTRACT

Integrin alpha 11 (Itga11) is the most recently identified integrin subunit and has been associated with fibrotic disease. To investigate the role of Itga11 in fibroblast behavior in wound healing and fibrosis we used *Itga11* null mice. We have found that fibroblasts lacking Itga11 (KO) repair a scratch in a confluent monolayer faster than their normal (Het) counterparts, and show increased proliferation along the margins of the scratch. In an attached collagen matrix model of fibrosis the KO fibroblasts show decreased expression of Col1A1 and αSMA, two markers of activated fibroblasts. However we did not find altered expression of these genes in a healing wound. In attached collagen gels KO fibroblasts exhibit decreased MMP13 collagenase activity, and in healing wounds blood vessels are abnormally distributed. Taken together these data suggest that Itga11 is involved in remodeling of the extracellular matrix and is required for the developing blood vessels to penetrate the wound bed.

15. SUBJECT TERMS

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Introduction

Scleroderma is characterized by fibrosis, the replacement of healthy tissue with collagenous matrix (Kissin, 2003). Fibroblasts synthesize and maintain connective tissue, and under normal conditions the cells remain relatively quiescent. In contrast, fibrotic conditions are characterized by activated fibroblasts that synthesize increased amounts of collagen, and generate contractile forces (Kissin, 2003). Fibroblast behavior during wound healing is similar to that observed in fibrosis and fibrosis has been characterized as an aberrant wound healing process (Darby, 2007; Hinz, 2010). The main receptors for the extracellular matrix (ECM) are the integrins, a large family of proteins that consist of heterodimeric transmembrane receptors composed of an alpha (α) and beta (β) subunit (Hynes, 2002). The collagen binding integrins are a subset of alpha chains namely α1, α2, α10 and α11, that pair with the β1 subunit (Gullberg, 2002; White, 2004), and all, with the exception of α 10, are expressed in fibroblasts (Popova, 2004). Integrin α11 (Itga11) is the most recently identified of the collagen binding integrins (Gullberg, 1995; Lehnert, 1999; Velling, 1999). Initial data has suggested a role for Itga11 in fibroblast function and force transduction (Popova, 2004), and over-expression has been associated with fibrotic conditions (Gardner, 2004).

Integrins interact with components of the ECM and generate intracellular signals (Hynes, 2002). Itga1 stimulates proliferation (Pozzi, 1998; Gardner, 1999) and decreases collagen production (Ekholm, 2002; Gardner, 1999; Riikonen, 1995), while Itga2 increases both collagen production and turnover (Ekholm, 2002; Gardner, 1999; Ivaska, 1999; Langholz , 1995; Riikonen, 1995). Using the contraction of collagen lattices *in vitro* Itga2 has been shown to be more important in force transduction than Itga1 (Cooke, 2000; Ravanti, 1999; Riikonen, 1995; Schiro, 1991). The control of collagen production and turnover is dsyregulated in fibroblasts from scleroderma lesions (Klein, 1991), suggesting that the diseased fibroblasts are less able to detect and respond to collagen in the ECM. Investigations into the roles of Itga1 and Itga2 in fibroblast function have indicated that their expression is altered in scleroderma (Eckes, 1996; Ivarsson, 1993; Kozlowska, 1996) but neither of these integrins has been conclusively linked to development of the disease.

Itga11 is highly expressed in mouse embryonic fibroblasts where it is localized to focal adhesions (Popova, 2004). Our preliminary data showed that Itga11 was also strongly expressed and localized in focal adhesions in adult dermal fibroblasts. In addition *Itga11* null fibroblasts are deficient in contracting floating matrices consisting of collagen type I (Popova, 2007; our unpublished data). We hypothesized that Itga11 expression is elevated in the activated fibroblasts known as myofibroblasts that are present during wound healing and scar formation (Hinz, 2010), and is required for the activated myofibroblast phenotype. To investigate the role of Itga11 in activated fibroblasts we used primary dermal fibroblasts derived from *Itga11* null (KO) mice. We investigated behaviors in cells derived from Het and KO mice using 2-dimensional and 3-dimensional models of wound healing *in vitro* and were able to perform two *in vivo* studies of wound healing.

Body

The heterozygous (Het) phenotype of one intact *Itga11* allele and one null allele appears indistinguishable from the wild-type (WT). Due to the ongoing problems with breeding the *Itga11* null (KO) mice, and small litter sizes on the C57BL/6 genetic background, we have maintained breeding pairs as Het paired with KO, thus only generating Het and KO littermates. It is important that primary cells are derived from mice of the same sex, and our previous experience has shown that cells isolated from female mice grow faster and survive longer in culture than cells derived from male mice. For the experiments presented here we have used Het and KO cells derived from female mice. While it would be preferable to compare WT and KO, breeding problems have made this impossible.

In vitro studies in two-dimensional culture.

Integrins have important roles in cell proliferation and migration during wound healing (Lee, 2004). We determined if the absence of Itga11 affected the rate of proliferation in cultured dermal fibroblasts. Equal numbers of Het and KO cells were seeded into 96 wells and the number of cells present measured after 24 and 48 hours in culture. Several assays are available to measure cell numbers, we chose to use the Cyquant fluorescent assay from Invitrogen. Cyquant dye fluoresces strongly when bound to double stranded DNA and the fluorescence is linear over a large range of cell numbers. Using this assay we have not observed any difference in the overall rate of proliferation between Het and KO fibroblasts (figure 1).

Proliferation of subconfluent cultures of cell *in vitro* may not be representative of cell behavior during wound healing. We have used an *in vitro* 'scratch' assay to investigate the role of Itga11 in a model that more closely resembles a wound healing situation. Primary dermal fibroblasts isolated from Het and KO mice were cultured to confluence on chamber slides. Using a sterile pipet tip the confluent monolayer of cells was scratched to create an *in vitro* model of a wound. The scratches were photographed immediately after creation and after 24 and 48 hours to monitor the rate at which the scratch was repopulated. Figure 2 shows images from a representative experiment. At 24 hours the cells at the edge of the scratch have started to migrate into the empty area (panels C and D), although the area of the scratch contains fewer cells in the Het cultures than the KO. After 48 hours cultures of KO cells have entirely filled in the scratched area (panel F). In contrast, the scratched area in the Het cells is still clearly visible at 48 hours.

Repopulating the denuded area likely involves both migration and proliferation. Figure 1 shows that in standard subconfluent cultures of cells we did not observe differences in the overall rate of proliferation between Het and KO cells. However cellular responses could be different along the margins of the scratch. We determined the rate of cell proliferation along the edges of the scratch using the thymidine analog 5-bromo-2-deoxyuridine (BrdU). BrdU is incorporated into DNA as it is being replicated, thus it is present only in cells that have undergone mitosis during the period of the BrdU pulse. BrdU was added to the cultures for the final 4 hours of incubation and positive nuclei visualized using a BrdU specific antibody. The number of BrdU positive

nuclei and the total number of nuclei (labeled with DAPI (4',6-diamidino-2-phenylindole)) were counted in 6 fields along the edge of the scratch, and the number of BrdU positive nuclei expressed as a percentage of the total nuclei in each field. Figure 3 shows that the percentage of nuclei positive for BrdU is higher in the KO cultures than the Het cultures. Taken together with the pictures in figure 2, these data show that KO fibroblasts can repopulate a denuded area faster than normal fibroblasts. At least a part of this effect can be explained by the fact that, compared with the Het cultures, a higher percentage of the KO cells adjacent to the scratch are actively dividing.

In vitro studies in floating collagen matrices.

In vivo, fibroblasts exist in a three-dimensional matrix with the majority of cellular contacts being made with the extracellular matrix (ECM) and few direct cell contacts. To more closely replicate these conditions *in vitro,* fibroblasts can be cultured in gels of collagen type I. In floating (unstressed) collagen gels fibroblasts remodel and contract the initially loose matrix of collagen fibrils into a dense tissue like structure. As they remodel the collagen fibers in the lattice, fibroblasts decrease expression of collagen, increase expression of interstitial collagenase (MMP-1 in humans, MMP-13 in mice), and eventually become quiescent and undergo apoptosis (Grinnell, 2003). In our preliminary data we showed that KO fibroblasts were deficient in their ability to contract floating collagen gels even when stimulated with the profibrotic cytokine TGFβ (data not shown). We have compared gene expression in fibroblasts cultured on plastic and in floating collagen gels with and without TGFβ treatment.

Previous data suggested that Itga11 was overexpressed in fibrotic tissues. Therefore we first tested whether expression of Itga11 was increased by the addition of TGFβ to fibroblasts *in vitro*. Expression analysis by QRT-PCR showed that when cultured on plastic or in collagen gels, addition of TGFβ resulted in increased Itga11 expression compared to the untreated cells (Figure 4). The degree to which Itga11 expression was increased of varied significantly between different isolates of cells, and between different individual experiments, indicated by large error bars. In addition it is not possible to directly compare levels of expression between the different culture systems. Thus, while we are confident that TGFβ causes increased Itga11 expression in fibroblasts cultured both on plastic and in floating collagen gels, we are unable to draw conclusions about the effects of culture system on the expression of Itga11.

Fibrotic conditions are characterized by replacement of healthy tissue by collagen type I produced by activated fibroblasts. Therefore we determined expression of collagen type I alpha 1 chain (Col1A1) in mouse primary dermal fibroblasts isolated from Het and KO mice, cultured on plastic and in floating collagen gels in the presence and absence of TGFβ. Over multiple experiments when TGFβ was added to fibroblasts cultured on plastic we did not observe any increase in expression of collagen type I in either the Het or KO fibroblasts (Figure 5, black bars). When fibroblasts were cultured in collagen gels we found that addition of TGFβ did result in increased expression of collagen type I (as expected). However, there was no appreciable difference in the expression of collagen type I between the Het and KO cells (Figure 5, red bars).

The data presented in figure 5 are fold change in expression normalized to the expression in the untreated Het cells in the two different culture systems.

In addition to elevated expression of collagen type I, the expression of alpha smooth muscle actin (αSMA) is currently the most accepted marker of activated fibroblasts (Hinz, 2010). The expression of αSMA allows activated fibroblasts to be contractile which contributes to contraction in healing wounds and the tension fibrosis (Gabbiani, 2001). KO fibroblasts are deficient in their ability to contract collagen gels. Therefore we examined the expression of αSMA in KO and Het fibroblasts. Figure 6 shows that αSMA expression was not altered in the KO fibroblast compared to the Het cells, and it's expression was increased to the same degree in response to addition of TGFβ, both on plastic and in collagen gels.

The accumulation of collagen depends on the balance of collagen production and breakdown. Likewise, the ability of fibroblasts to contract collagen gels requires both their ability to contract, through expression of αSMA, and the breakdown of extracellular matrix (ECM) components (Grinnell, 2003). MMP13 is a major proteinase for collagen type I (Ravanti, 1999). Because we did not observe changes in Col1A1 or αSMA expression, we next examined expression of MMP13. Initial data suggested that MMP13 expression was lower in KO fibroblasts compared to the Het (data not shown). However, subsequent experiments did not bear out the original findings, and figure 7 shows MMP13 expression does not appear to be different in KO cells compared with Het cultured on plastic or in floating collagen gels.

In vitro studies in attached (stressed) collagen matrices.

In a floating collagen gel the cells are able to freely remodel the matrix. In contrast, when the collagen matrix remains attached to the walls of the well (stressed) the fibroblasts become aligned with the plane of the restraint, and assume a myofibroblast-like phenotype, similar to that seen in healing wounds (Grinnell, 2003). We investigated gene expression in Het and KO fibroblasts cultured in attached collagen matrices, examining the same set of genes described for the floating collagen gels. One hallmark of fibrotic conditions is the accumulation of collagen type I. In the floating collagen gels we did not observe any difference in expression of collagen type I in the KO fibroblasts (figure 5). In contrast, when cultured in attached collagen matrices we found that in the presence of TGFβ the Het fibroblasts exhibited increased collagen production while the KO cells did not. In the absence of TGFβ there was no apparent difference in expression of collagen type I between the Het and KO fibroblasts although the cells are under tension in this system. This data suggests that Itga11 may be required for fibroblasts to increase collagen type I expression in activated fibroblasts directly in response to TGFβ, but that other signals may compensate.

In addition to regenerating the dermis, fibroblasts in a healing wound also generate tensile forces that contribute to contraction of the wound site and help to bring the epithelial margins together. The expression of αSMA and its localization to stress fibers is thought to be required for the contractile ability of myofibroblasts (Gabbiani, 2003; Hinz, 2010), and αSMA expression

has been correlated with fibrosis. We found that in attached collagen gels the expression of αSMA was decreased in the KO fibroblasts compared to the Het cells, both in the presence and absence of added TGFβ (figure 9). We were not able to measure the degree of contraction of the attached gels, but this data suggests that fibroblasts require Itga11 to upregulate expression of αSMA when exposed to tensile forces.

We also examined the expression and activity of MMP13 in the attached collagen gels. MMP13 is important for the turnover of matrix as fibroblasts remodel their environment. A decrease in MMP13 could lead to an accumulation of collagen in the ECM even if there were no increase in collagen expression. QRT-PCR of fibroblasts cultured in attached gels suggests that expression of MMP13 may be decreased in KO cells under stressed conditions (figure 10). To further investigate MMP13 expression, and also activity, we performed a western blot and zymogram analysis on conditioned medium taken from cultures of fibroblasts in both attached and floating collagen gels. MMP13 is initially expressed as an inactive zymogen, and its activity is regulated in the extracellular milieu. We analyzed the total amount of MMP13 present in the conditioned medium by western blot using an antibody that detects both the inactive zymogen and the smaller molecular weight active species. Figure 11 A shows a representative western blot with a strong band detected at about 60kD, which corresponds to the inactive form of the enzyme. We did not detect any signal at the lower mw that would correspond to the active form of the enzyme. In this and one other independent experiment there were no difference in the intensity of the band at 60 kD in conditioned medium from floating or attached collagen gels. These data suggest that any apparent changes in the expression of MMP13 detected by QRT-PCR (figure 10) did not translate into changes in the amount of secreted protein. The activity of MMP13 is controlled in the extracellular environment by cleavage of a propeptide to produce an active enzyme. To measure the activity of MMP13, samples of conditioned medium were separated on a native gel containing 0.1% gelatin which acts a substrate for activated MMP13. In areas where active collagenases are present the gelatin in the gel is digested and these areas will appear white against the blue background of the subsequently stained gel. Figure 11 B shows a representative zymogram of conditioned medium from cells cultured in floating and attached collagen gels. In attached collagen gels enzyme activity is less in the samples of conditioned medium from KO fibroblasts than the corresponding Het samples. In the samples from the floating collagen gels there does not appear to be any difference in enzyme activity between the samples. These data suggest that while there may not be alterations in the expression of MMP13 in the KO fibroblasts, the activity of this enzyme is decreased compared to the Het cells cultured under the same conditions.

In vivo model of excisional wound healing

In a preliminary wound healing experiment 5 mm full thickness excisional wounds were created on the lower back of six female Het and KO littermates. Mice were observed daily over the course of the experiment and sacrificed at 6 days (n=3) and 12 days (n=3) following wounding. No gross changes in the rate of wound healing were observed between the Het and KO mice over the course of the experiment. We examined sections of formalin-fixed tissue for expression of collagen type I but did not observe any overall differences in expression levels

between the two genotypes (data not shown). We also looked at the expression of αSMA as a marker of activated myofibroblasts. While there were no differences in the overall level of αSMA in the granulation tissue, we did observe a change in the pattern of αSMA expression. In addition to being a marker of myofibroblasts, αSMA is also expressed in smooth muscle cells associated with blood vessels. Thus, αSMA expression in the granulation tissue indicates the position of the newly formed blood vessels. In the sections from KO animals the αSMA positive structures appeared to be clustered close to the dermo-epidermal junction, while in the Het samples they were distributed evenly throughout the granulation tissue (Figure 12A). To confirm the pattern of blood vessels, sections of granulation tissue were immunostained for CD31 (PECAM) a specific marker of endothelial cells (data not shown) and the number of CD31 positive structures were counted in the first 100 µm strip directly under the epidermis (figure 12B). At day 6 following wounding there were significantly more blood vessels located close to the dermo-epidermal boundary in the KO mice compared with the Het controls ($p=0.02$). By day 12 there was no longer a difference between the two. A second study with a larger number of mice looking at 6 days after wounding confirmed the clustering of blood vessels at the dermo-epidermal junction in the KO mice. Figure 13A shows representative images of the CD31 immunostain in the wound bed. CD31 positive structures were counted in 50 µm 'strips' throughout the granulation tissue (figure 13B), confirming the clustering of vessels just under the epidermis in the KO mice.

Key research accomplishments

- We have not observed differences in the overall rate of proliferation between Het and KO fibroblasts.
- KO cells repopulate a scratched area faster than Het fibroblasts.
- This can be partly explained by higher rates of cell proliferation observed along the margins of the scratch in the KO cultures.
- We have used the floating collagen gel system to culture fibroblasts in an environment that is closer to the *in vivo* conditions of normal skin.
- In the floating collagen gels we have found that the absence of Itga11 expression does not result in any difference in the expression of collagen type I, αSMA or MMP13, all of which are involved in wound healing and have been implicated in fibrosis.
- We have used the model of attached, or stressed, collagen gels to investigate gene expression in a system that more closely mimics a fibrotic condition.
- Expression of collagen type I and αSMA were both reduced at the RNA level in KO fibroblasts cultured in attached collagen gels compared with the Het controls.
- Expression of MMP13 in the attached collagen gels appeared slightly lower at the RNA levels, but a western blot detected equal amounts of protein secreted from the cells.
- Zymogram analysis of MMP activity showed less collagenase activity in the KO cells cultured in the attached gels, compared to the Het controls. In the floating, unstressed gels there were was no difference in MMP13 activity.
- In an *in vivo* model of healing of full thickness excisional wounds there were no gross healing defects in the KO mice.

- Immunohistochemical analysis of the healing wounds showed that at early stages of healing (6-days) there was an abnormal distribution of blood vessels in the granulation tissue of the KO mice. In the KO mice the vessels clustered close to the epidermal boundary at 6 days after wounding, but this effect was no longer seen at 12 days after wounding.

Reportable outcomes

There are currently no reportable outcomes.

Conclusion

Task 1. Studies in two-dimensional culture

Cell culture studies on tissue culture plastic have shown that there is no overall difference in proliferative rate between KO and Het fibroblasts. However, in a scratch model where confluent monolayers of cells are damaged to create a scratch, KO fibroblasts fill in the denuded area more quickly than their Het counterparts. This can in part be explained by the higher rates of proliferation along the edge of the scratch that were found in the KO cells. Itga11 is localized in focal adhesions which are involved in the contacts between cells and their matrix. In order for cells to fill in the scratch they need to migrate and proliferate to increase cell numbers. It is likely that the absence of Itga11 makes it easier for the cell to detach from the matrix thereby increasing the rate of migration and also proliferation.

Task 2. Studies in unstressed floating collagen gels.

In floating (unstressed) collagen gels fibroblasts remodel and contract the initially loose matrix of collagen fibrils into a dense tissue like structure. As they remodel the collagen fibers in the lattice, fibroblasts decrease expression of collagen, increase expression of interstitial collagenase (MMP-1 in humans, MMP-13 in mice), and eventually become quiescent and undergo apoptosis (Grinnell, 2003). We, and others (Popova, 2007), have found that KO fibroblasts have a defect in their ability to contract these floating collagen gels. We used this system to investigate the expression of genes commonly associated with wound healing and the development of fibrosis. In floating collagen gels we did not observe any differences in expression of collagen type I, αSMA or MMP13 in the KO fibroblasts compared with Het cells. The floating collagen matrices are thought to provide a cellular environment that mimics the resting dermis. No abnormalities have been found in the skin of KO mice, therefore it is perhaps not surprising that we did not find changes in expression of genes that are responsible for the maintenance of the extracellular matrix.

Task 3. Studies in stressed attached collagen gels

When collagen gels remain attached to the walls of the well the fibroblasts within the matrix become aligned with the plane of the restraint, and assume a myofibroblast-like phenotype,

similar to that seen in healing wounds (Grinnell, 2003). In this model we found that both collagen type I and αSMA expression were reduced in the KO fibroblasts. These data were obtained with QRT-PCR examination of the level of expression at the RNA level and we did not have sufficient cells to confirm these data at the protein level. Examination of MMP13 in the stressed gels suggested a decrease in MMP13 expression in the KO fibroblasts, but this was not confirmed by a western blot of conditioned medium, which showed equal levels of protein expressed under different conditions. However we did find that the activity of MMP13 was reduced in conditioned medium from KO fibroblasts cultured in attached collagen gels compared with the controls. These data suggest that Itga11 may be involved in pathways that lead to fibroblast activation under stressed conditions, and that in particular it is involved in remodeling of the extracellular environment through the expression and regulation of MMP13 activity.

Task 4. Examination of wound healing in KO mice.

In two experiments we did not find any gross differences in cutaneous excisional wound healing between KO mice and Het controls. Immunohistochemical analysis did not find any differences in the amount of collagen type I or αSMA in the wound bed. We did find an altered pattern of αSMA expression suggesting a disrupted pattern of blood vessel distribution through the granulation tissue. In the KO 6-day wounds the αSMA positive structures were localized close to the dermo-epidermal junction instead of being evenly distributed through the granulation tissue. We confirmed that the αSMA positive structures were blood vessels by immunostaining with CD31 (PECAM), a specific marker of endothelial cells. By 12 days after wounding blood vessels were evenly distributed in the granulation tissue of the KO mice. Taken together with the data from the attached collagen gel system showing decreased MMP13 activity in KO fibroblasts, we conclude that Itga11 has a role in remodeling the extracellular matrix to allow penetration of developing blood vessels. *In vitro* data from the attached collagen gel system suggests that Itga11 may also have a role in the activation of fibroblasts to myofibroblasts through expression of collagen type I and αSMA. However, we did not find any evidence of a defect in myofibroblast differentiation in healing wounds.

The data generated in this project suggest that Itga11 has a minor role in the activation of myofibroblasts. Under stressed conditions *in vitro* KO fibroblasts showed decreased MMP13 collagenase activity compared to control fibroblasts. This decreased MMP13 activity *in vitro* correlates with a disorganization of blood vessels at the early stages of wound healing in an excisional cutaneous wound where the developing blood vessels need to penetrate through the granulation tissue. Any other changes in KO fibroblasts that were observed *in vitro* were apparently overcome *in vivo* as wound healing progressed otherwise normally in the KO animals. We did not find any decrease in the expression of collagen type I or αSMA, two markers activated fibroblasts, in the granulation tissue from KO animals. Therefore it is unlikely that Itga11 will make an attractive target for the treatment of fibrotic conditions.

This project was hampered by poor breeding of the *Itga11* null colony. We decided to cross the *Itga11* null allele onto the C57BL/6 genetic background because this is most commonly used to

investigate skin diseases. In general the C57BL/6 mice are poor breeders and it can take several pregnancies to get a litter that survives to weaning. We had worked with C57BL/6 mice previously at different institutions and had not found these issues to be insurmountable. We took many measures to try and improve the size of the colony, such as males being removed from the cage when females were pregnant, supplying breeding females with enriched breeder chow and mating females whose previous litters had not survived. Despite these measures, many females that got pregnant did not care for their litters and there also appeared to be a problem with females getting pregnant. This could have been due in part to the intensive building work that has been ongoing at UCLA in the vicinity of the animal facility, and anecdotally I heard of several other people having problems with their mouse colonies. Breeding problems meant that the supply of mice for both *in vivo* and *in vitro* experiments was limited, and we were only able to perform two small *in vivo* wound healing studies. It was not feasible to cross the KO mice with the TβRIIΔK transgenic mouse in which expression of a mutated TGFβ receptor in fibroblasts results in skin and lung fibrosis *in vivo*.

Personnel on project

Ruth M Baxter, principal investigator
Sarah Garbers, research associate

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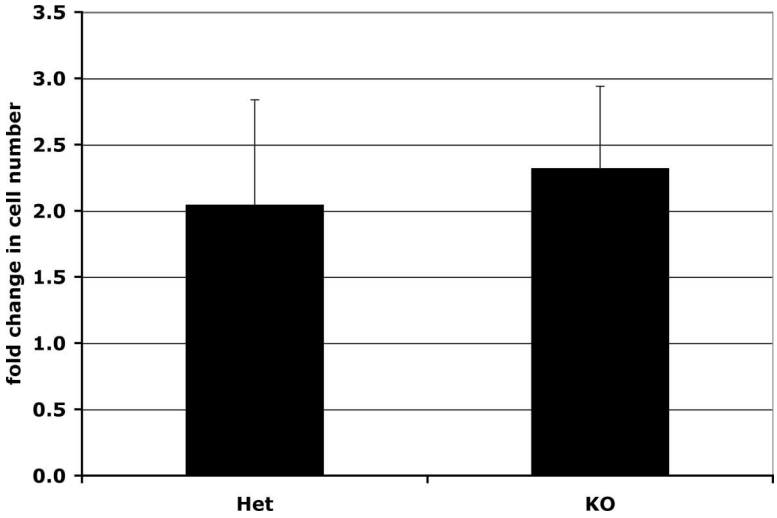

Figure 1. Proliferation rate of Het and KO dermal fibroblasts. Het and KO fibroblasts were seeded into 96 wells at $1.5x10^3$ cells per well in 100 µl of DMEM plus 10% FBS. Cell numbers were measured after 24 and 48 hours using the Cyquant assay (Invitrogen). Cyquant dye fluoresces strongly when bound to double stranded DNA and therefore gives an accurate representation of the number of nuclei present in each well. Medium was aspirated and dye added in Hanks Buffered Salt Solution (HBSS). Cells were incubated with the dye for 30 minutes before fluorescence was measured using a SpectraMax plate reader with excitation at 485nm and emission at 530nm. Data presented are the mean and standard deviation of the fold change in cell number between 24 and 48 hours for three independent cell isolates of paired Het and KO mice.

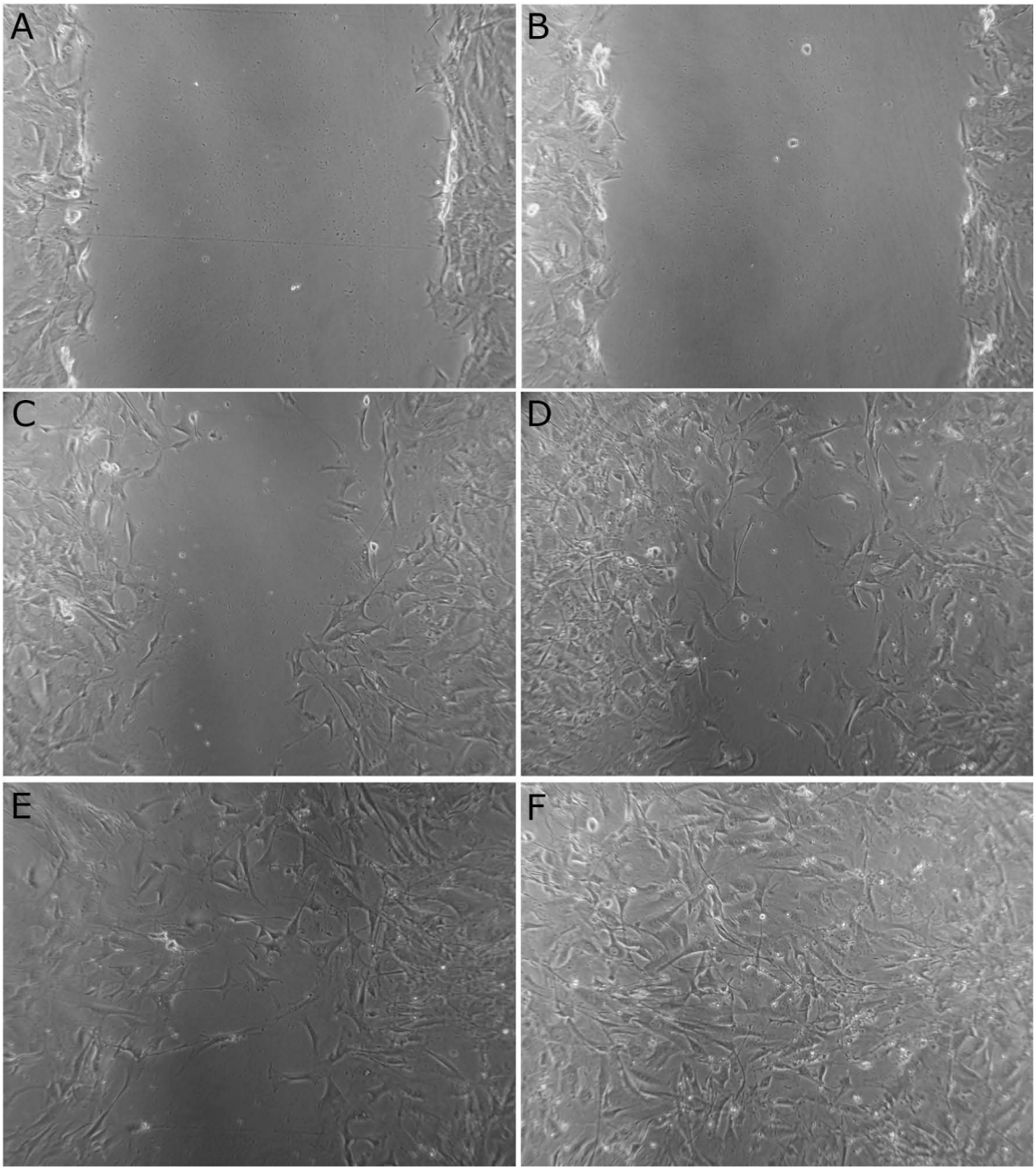

Figure 2. Fibroblast repopulation of *in vitro* scratch. Primary dermal fibroblasts isolated from Het (A, C, E) or KO (B, D, F) mice were seeded into 4-well chamber slides at a density of $5x10^4$ cells/well in a volume of 1 ml DMEM containing 10% FBS. The following day a sterile pipet tip was used to make two scratches across the well perpendicular to each other forming a cross in the confluent cell layer. After forming the scratch the medium was aspirated, the monolayer gently washed with fresh medium and 1ml fresh serum containing medium added. The area of the scratch just above the cross was photographed immediately (A, B) and after 24 hours (C, D) and 48 hours (E, F). Pictures are from a single experiment that is representative of three independent experiments.

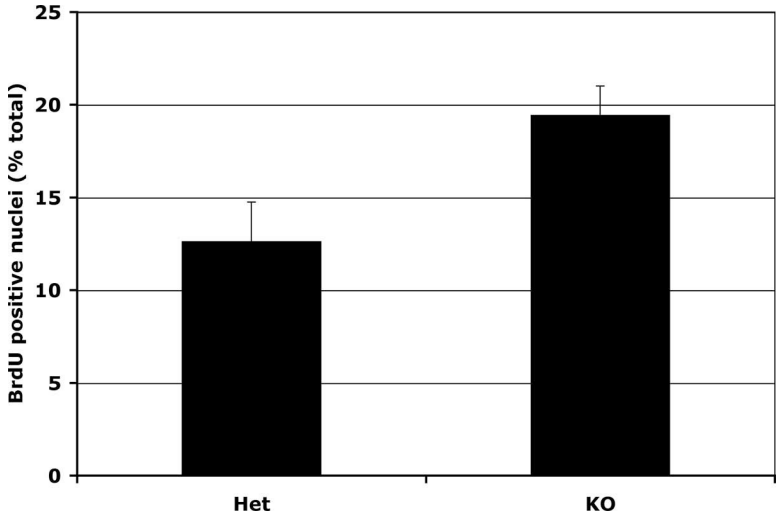

Figure 3. **Proliferation of cells adjacent to the scratch**. 10µM BrdU was added to the scratch assay cultures for the final 4 hours of the 48 hr incubation. Cells were fixed with 3% paraformaldehyde followed by incubation with a BrdU specific antibody (Bioegend, clone MoBU-1 mouse IgG1) and a secondary anti-mouse antibody conjugated with Alexa-594. To non-specifically label nuclei DAPI (4',6-diamidino-2-phenylindole) at a final concentration of 2 µg/ml was added with the secondary antibody. Samples were mounted with an a aqueous fluorescent mounting medium and photographed with a Nikon Eclipse E 800-S fluorescence microscope. The number of BrdU positive nuclei (red) and the total number of nuclei (blue) were counted in 6 fields along the edge of the scratch, and the number of BrdU positive nuclei was expressed as a percentage of the total nuclei in each field. Data presented are the mean and standard deviation from three independent experiments.

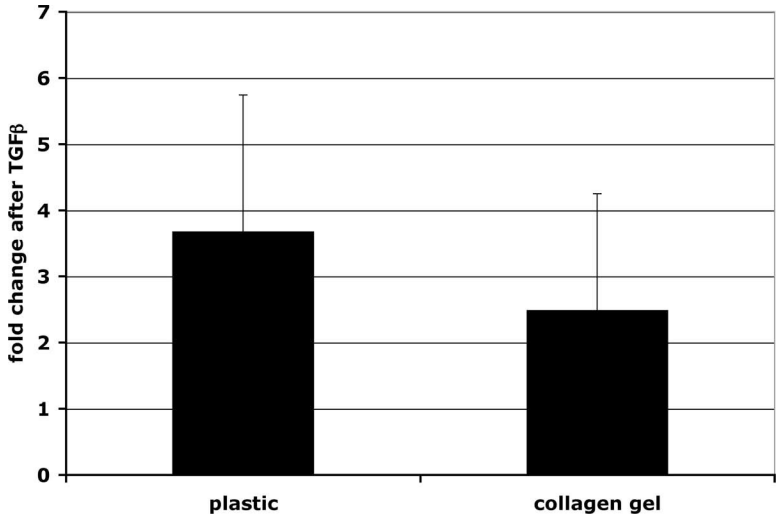

Figure 4. Itga11 expression in dermal fibroblasts cultured on plastic or in floating collagen gels treated with TGFβ. Het fibroblasts were cultured on plastic ($5x10^4$ cells per well of 6-well plate) or in floating collagen gels ($1x10^6$ cells/ml of 1.5mg/ml bovine collagen type I in DMEM) for 48 hours in the presence or absence of 10 ng/ml TGFβ (R&D Systems). Total RNA was isolated using mini spin columns (Omega Biotek) and 0.5µg RNA used to synthesize cDNA (cDNA syntheis kit, Quanta Biosciences). QRT-PCR reactions were carried out using Sybr green detection (Sybr Fastmix, Quanta Biosciences) using a Stratagene MX300P system. Expression of Itga11 was normalized to the expression of a control gene, Rpl13, in each sample. Primer sequences used were: Itga11 forward: TGAACACTGTGTCCCTGACC, Itga11 reverse: CGCGTGCTCTCTATGATGAA, Rpl13 forward: TTTTGCCAGTCTCCGAAT, Rpl13 reverse: TGCTTTATGGAAAATTTATTGC. For each individual experiment the fold change in Itga11 expression after TGFβ treatment was calculated and data presented are the mean and standard deviation of 4 independent experiments.

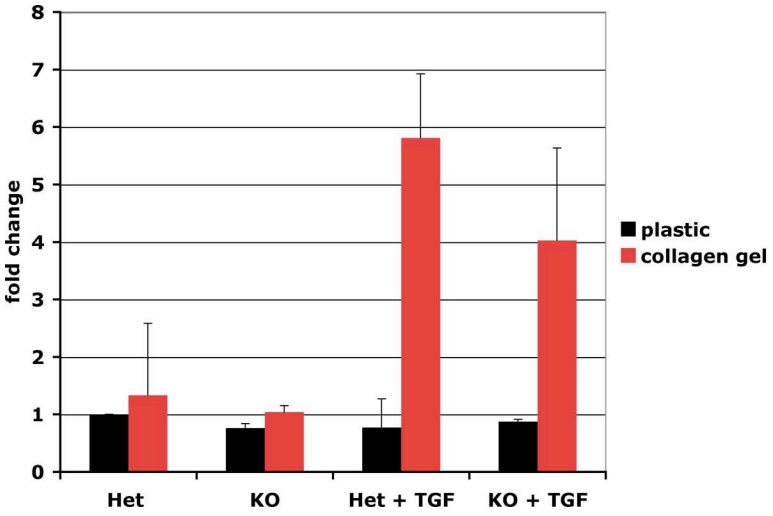

Figure 5. Expression of collagen type I in dermal fibroblasts cultured on plastic or in floating collagen gels. Cells were cultured as described in the legend to figure 4. Specific primer sequences for the alpha1 chain of collagen type I were forward: GTATTGCTGGACAACGTGGT and reverse: AATGCCTCTGTCACCTTGTTC. Expression of collagen type I was normalized to expression of Rpl13 in each sample. For each experimental condition (culture on plastic (black bars) and in collagen gels (red bars)) the normalized expression of collagen type I was compared to the expression in the Het fibroblasts untreated with TGFβ. Data presented are the mean and standard deviation of 2 independent experiments.

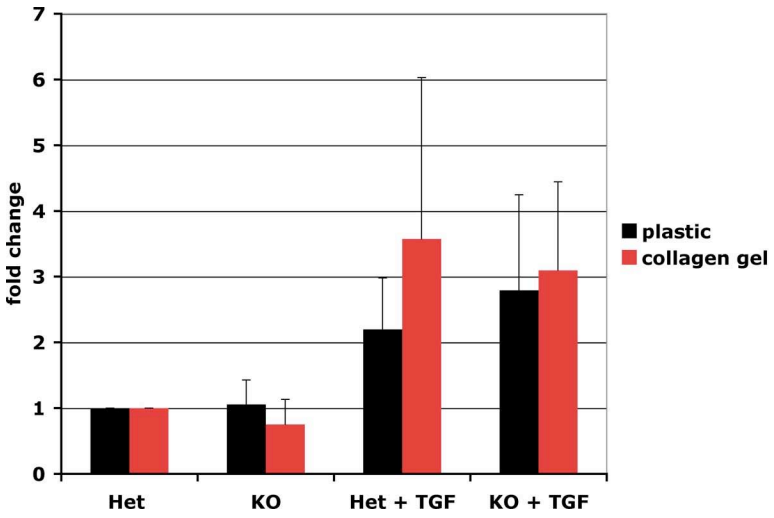

Figure 6. Expression of αSMA in dermal fibroblasts cultured on plastic or in floating collagen gels. Cells were cultured as described in the legend to figure 4**.** Specific primer sequences for αSMA were forward: ATGTGTGAAGAGGAAGACAGCA, and reverse: GCCGTGTTCTATCGGATACTTC. Expression of αSMA was normalized to expression of Rpl13 in each sample and compared to the expression in the Het fibroblasts untreated with TGFβ for each culture condition. Data presented are the mean and standard deviation of 2 independent experiments.

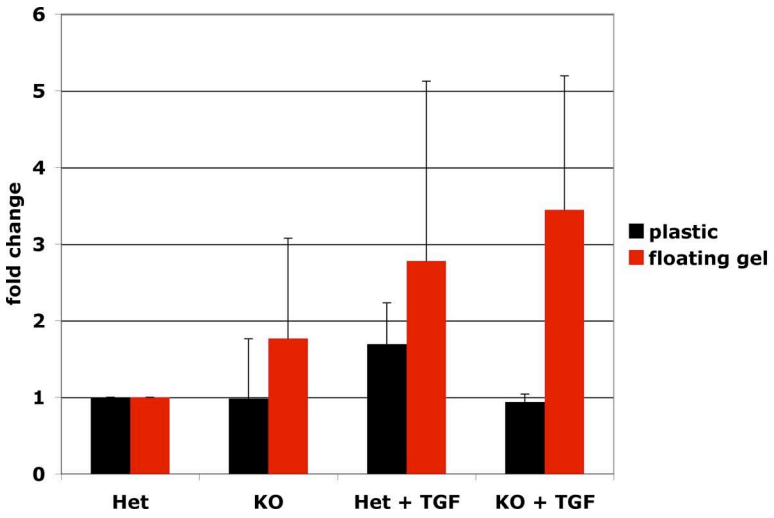

Figure 7. Expression of MMP13 in dermal fibroblasts cultured on plastic or in floating collagen gels. Cells were cultured as described in the legend to figure 4. Specific primer sequences for MMP13 were forward: CATGAGAAAACCAAGATGTGGA and reverse: TCTGGTGAAATTCAGTGGTGTC. Expression of MMP13 was normalized to expression of Rpl13 in each sample and compared to the expression in the Het ctrl for each condition. Data presented are the mean and standard deviation from 4 independent experiments.

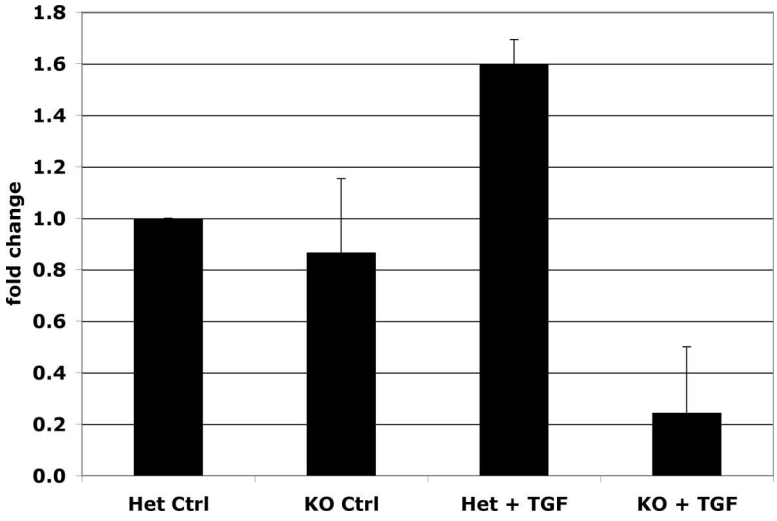

Figure 8. Expression of collagen type I in fibroblasts cultured in attached collagen gels. Het and KO fibroblasts were cultured in attached collagen gels ($1x10^6$ cells/ml of 1.5mg/ml bovine collagen type I in DMEM) for 48 hours in the presence or absence of 10 ng/ml TGFβ. RNA was extracted, cDNA synthezised and QRT-PCR reactions performed as described in the legend to figure 4. Expression of collagen type I was normalized to expression of Rpl13 in each sample and compared to the expression in the Het fibroblasts untreated with TGFβ. Data presented are the mean and standard deviation of 2 independent experiments.

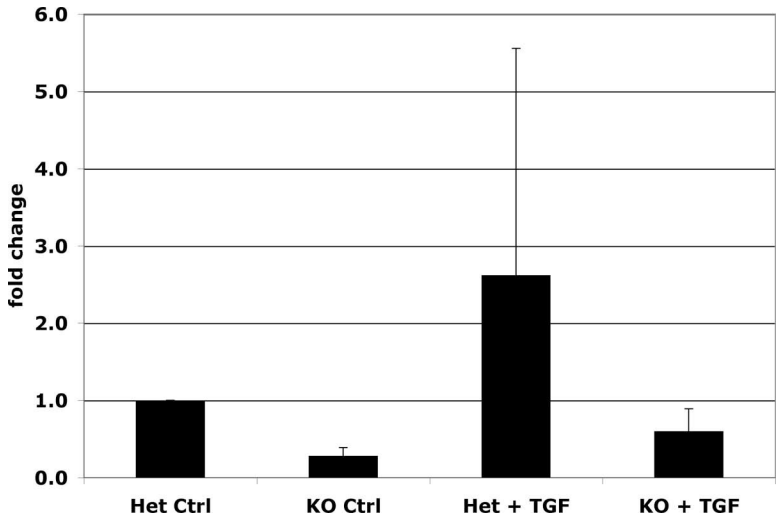

Figure 9. Expression of αSMA in fibroblasts cultured in attached collagen gels. Expression of αSMA in fibroblasts cultured in attached collagen gels was normalized to expression of Rpl13 in each sample and compared to the expression in the Het fibroblasts untreated with TGFβ. Data presented are the mean and standard deviation of 2 independent experiments.

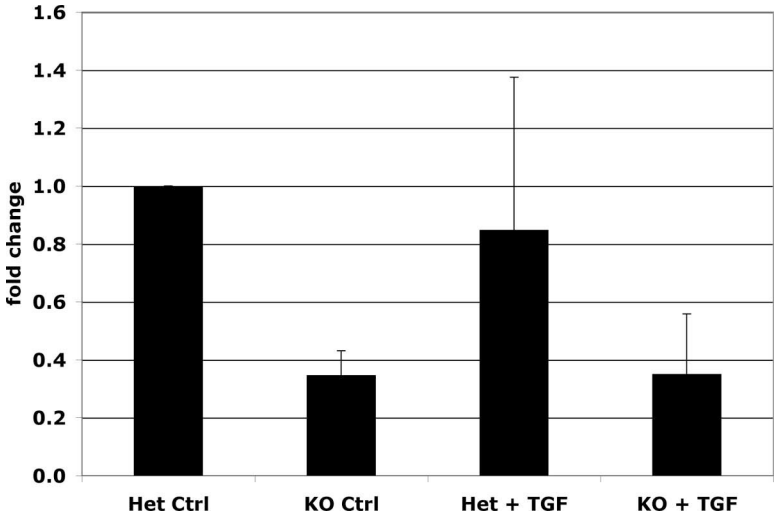

Figure 10. Expression of MMP13 in fibroblasts cultured in attached collagen gels. Expression of MMP13 was normalized to expression of Rpl13 in each sample and compared to the expression in the Het fibroblasts untreated with TGFβ. Data presented are the mean and standard deviation of 2 independent experiments.

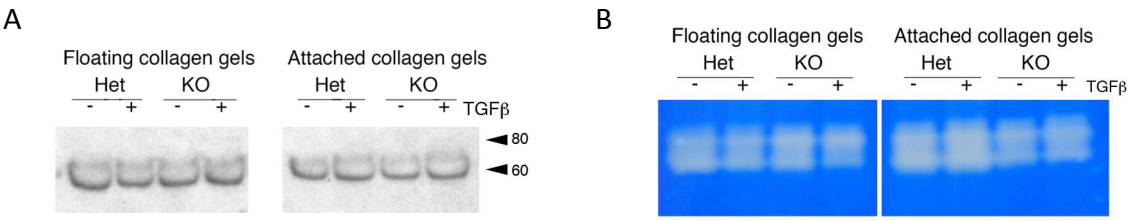

Figure 11. Expression and activity of MMP13 in fibroblasts cultured in floating and attached collagen gels. Equal numbers of Het and KO fibroblasts were cultured in floating and attached collagen gels for 48 hours in the presence or absence of 10 ng/ml TGFβ. Conditioned medium was collected and analysed for the expression of MMP13 by western blot (A) or for collagenase activity (B). A. For western blot detection, samples were separate by electrophoresis on a 4-12% bis tris gel (Novex, Invitrogen) and transferred to PVDF membrane. The membranes were probed with anti-MMP13 (Abcam ab39012) that recognizes the latent and active forms of MMP13, followed by an HRP (horse raddish peroxidase)-conjugated secondary antibody and detection using ECL (enhanced chemiluminescence). A large band was detected at 60kD which corresponds to the weight of the latent proenzyme. B. To detect the activity of MMP13 in the samples, equal volumes of conditioned medium were separated on native 10% tris glycine gels containing 0.1% gelatin (Novex zymogram gels, Invitrogen) as a collagenase substrate. Following electrophoresis gels were renatured and developed overnight at 37°C overnight, and areas of activity defined as unstained areas in a blue stained gel (Safestain, Invitrogen). Data shown are from a single experiment, similar data were found in one other experiment.

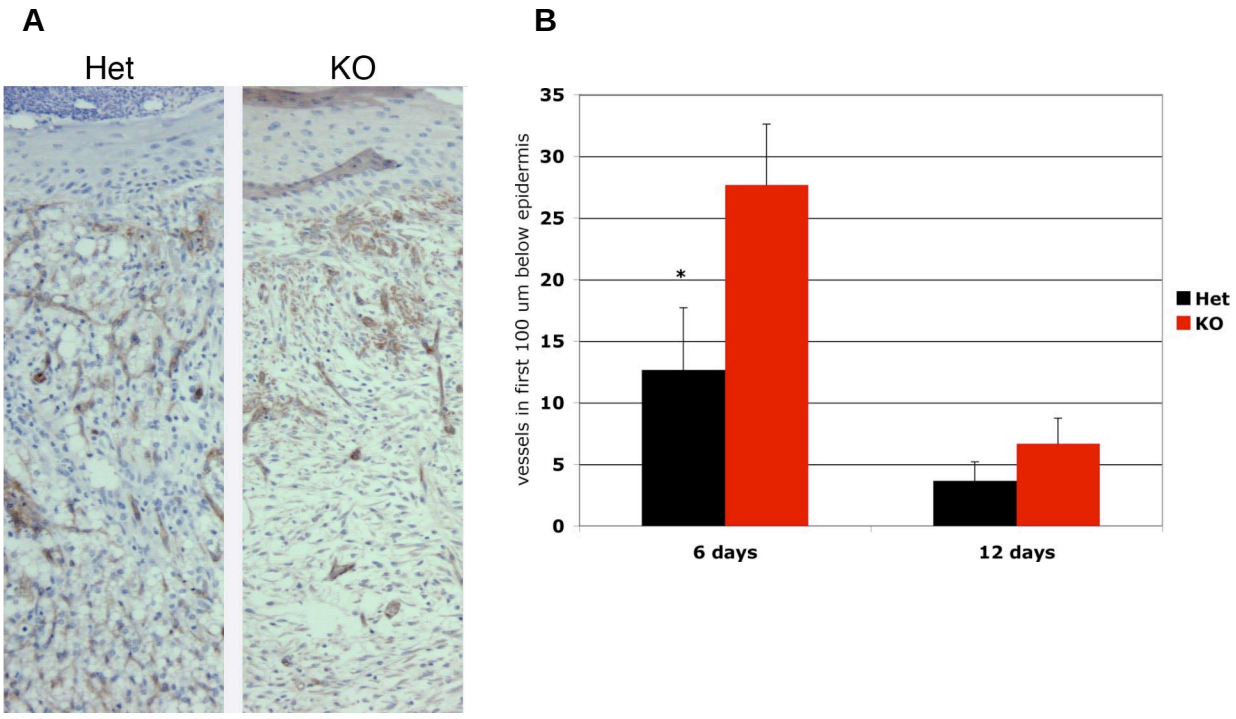

Figure 12. Distribution of αSMA positive structures in healing excisional cutaneous wounds. 5mm full thickness wounds were created on the backs of adult female KO mice (n=6) and Het littermate controls (n=6). Mice were euthanized 6 and 12 days (n=3) after wounding and tissue processed into paraffin blocks. (A) 7 µm sections were stained with antibodies against αSMA. In the KO mice at 6 days following wounding the blood vessels were not evenly distributed through the granulation tissue, but instead appeared to be clustered at the dermo-epidermal junction. (B) The number of blood vessels in the first 100 µm below the epidermis were counted in sections from 6 and 12 days after wounding. At 6 days the KO mice have significantly more vessels close to the dermo-epidermal junction. *p=0.02.

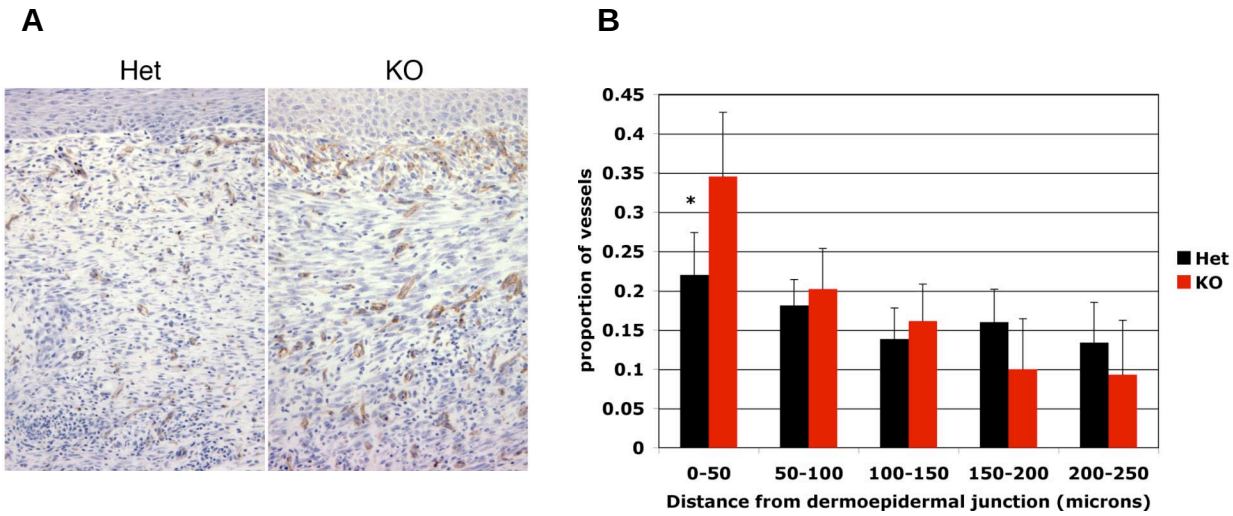

Figure 13. Distribution of blood vessel in healing excisional cutaneous wounds. 5mm full thickness wounds were created on the backs of adult female KO mice (n=9) and Het littermate controls (n=10). After 6 days mice were euthanized and tissue processed into paraffin blocks. (A) 7 µm sections were stained with antibodies against CD31 (PECAM) a marker of endothelial cells, to visualize blood vessels. In the KO mice at 6 days following wounding the blood vessels were not evenly distributed through the granulation tissue, but instead appeared to be clustered at the dermo-epidermal junction. (B) The number of blood vessels in defined areas was counted, and there was a significant difference in the proportion of blood vessels in the first 50 µm beneath the dermo-epidermal junction between wild type and Itga11 null. *p=0.00047.